

ORIGINAL ARTICLE

FoxP3, GATA-3 and T-bet expression in elderly asthma

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Summary

Background Asthma is a chronic inflammatory disorder in which Th2, Th1 and suppressive T cells (Tregs) play a role. The transcription factor FoxP3 plays a role in Treg differentiation while T-bet is important for Th1 and GATA-3 for Th2 differentiation from naïve T cells. Recent data show that age-related deregulation of Treg cells is a mechanism of senescence affecting several chronic diseases. It is crucial to understand the behaviour of these cell populations in asthma for elderly patients.

Objective To evaluate FoxP3, GATA-3 and T-bet gene expression under basal conditions and after *in vitro* activation in a group of elderly asthmatic compared with age-matched healthy individuals.

Methods Thirty-two elderly asthmatics and 17 healthy elderly individuals were selected. Serum total IgE was measured, and peripheral blood mononuclear cells (PBMCs) were isolated and stimulated *in vitro* with anti-CD3/anti-CD28, followed by mRNA isolation. After reverse transcription, real-time quantitative PCR was performed and relative quantification was determined $2^{-\Delta\Delta C_t}$ ($2^{-\Delta\Delta C_t}$ method).

Results The mean values and standard deviation of FoxP3, GATA-3 and T-bet relative expression for control vs. asthma were 10.2 ± 6.8 vs. 4.8 ± 3.8 , 2.4 ± 2.9 vs. 1.7 ± 0.9 and 3.3 ± 2.1 vs. 2.1 ± 1.5 , respectively. Healthy individuals showed significantly higher expression of FoxP3 and T-bet; asthmatics had a lower T-bet/GATA-3 ratio, higher serum IgE and a positive significant correlation between total IgE and GATA-3 expression.

Conclusion and clinical relevance Elderly asthmatic patients have lower FoxP3 mRNA expression in PBMC, which can be associated with the sustained inflammatory process and with the decreased immune tolerance by Treg cells. The T-bet deficiency and the correlation of GATA-3 expression with the increase of IgE are characteristics of long-lasting asthma. Changes related to the immunosenescence process could provide an explanation for the minor differences observed between the groups. It is important to clarify persistent modifications in long-lasting asthma in the elderly and adequate future therapeutic approaches.

Keywords aging, asthma, FoxP3, GATA-3, regulatory T cell, T-bet

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Introduction

Asthma is an airway chronic inflammatory disorder characterized by a decline in pulmonary function that is correlated with age, disease duration and severity. As more than 300 million individuals suffer from this disease and the prevalence is increasing [1], it is an important field of study. It is often reported in children but affects all age groups including the elderly [1, 2]. In this age group, this pathology represents a growing clinical problem, with

an estimated prevalence between 6% and 14%, and is characterized by the same clinical features of the younger population [3–6].

In asthmatic airways, Th2 cells are activated and release several cytokines that regulate IgE production and inflammatory cell recruitment, such as eosinophils [7]. Several studies addressed the role of Treg in allergic diseases preventing disease development by suppressing their activity such as Th2 cytokine production [8, 9]. Treg has been defined by markers including CD4, CD25, and

more recently, a member of the forkhead box transcription factor – FoxP3 – was set as a more specific marker and is also important for their development and function [10]. These cells have a suppressive effect on inflammatory responses and are recognized as a *major* cell subset maintaining peripheral immune tolerance [11–13]. FoxP3 is crucial for *naïve* T cell differentiation towards the Treg phenotype and is considered as the main regulator of CD25⁺ Treg cell activation [12, 14]. These cells prevent both activation and effector function of autoreactive T cells that escaped to other mechanisms of tolerance [13] and they are assumed to control not only pathogenic Th2 cells but also Th1 cells. Although Th2 cells have been the most frequently studied T cells in asthma, there is evidence that Th1 cells are also involved in the development of this disease. Moreover, Th2 and Th1 cells are regulated and committed by the transcription factors GATA-3 and T-bet, respectively.

Treg cells are essential in the regulation of inflammatory diseases and more data are needed to clarify their role in allergic diseases in addition to the established role of Th1 and Th2 cells. During the ageing process, the immunoinflammatory response is modified and it is assumed that in this age group peripheral blood (PB) Treg cells are increased [5, 15].

The aim of this study was to evaluate fold increase of FoxP3, GATA-3 and T-bet mRNA expression, in peripheral blood mononuclear cells (PBMCs), under basal conditions and following *in vitro* activation with anti-CD3/anti-CD28, considering the ability of generated signals to establish reciprocal modulation, in elderly asthmatic patients. This study intends to clarify whether the decrease on Treg cells reported in adult asthma persists in the elderly aged groups despite the increased levels of Treg cells inherent to the immunosenescence process.

Material and methods

Subjects and blood samples

This study enrolled 32 elderly (≥ 65 years) (mean age 72 ± 5 years) patients who had been suffering from asthma for > 30 years, after informed consent. All patients had early-onset asthma starting at 26 ± 11 years. The diagnosis and severity of asthma were defined according to guidelines of the Global Initiative for Asthma [1]. All patients had asthma, controlled by using 250–750 μg of beclomethasone dipropionate daily and short-acting β_2 -agonists as needed. All other anti-asthmatic drugs were withdrawn at least 4 weeks before the study. The control group consisted of healthy non-allergic elderly volunteers ($n = 17$) older than 65 years (mean age 78 ± 7 years). The absence of allergic diseases and asthma in this control group was confirmed on the basis of a detailed clinical questionnaire and skin prick tests (SPT).

Smoking subjects and patients with recent (last 6 weeks) infectious diseases, auto-immune diseases and neoplastic diseases were excluded.

Lung function was assessed by spirometry (Vitalograph Compact, Ennis, Ireland) at least 6 h after the last dose of any bronchodilator. Predicted values of forced expiratory volume in 1 s (FEV₁) were measured according to Knudson et al. [16]. Fractional concentration of exhaled nitric oxide (FENO) was measured using a NioxMino collection device (Aerocrine, Solna, Sweden) with an expiratory flux of 50 mL/s during 6 s, according to the American Thoracic Society recommendations [17]. All the individuals studied were subjected to SPT to 20 common aeroallergens (ALK-ABELLO/Lancetter-tames Hollister Stier). Histamine dihydrochloride was used as a positive control (10 mg/mL) and saline solution used as a negative control. Subjects were classified as allergic with one positive test associated with clinical symptoms and as atopic if they had at least one positive SPT.

The entire study was performed during January/February, outside the grass, weed and tree pollen season. In this period, house dust mites' growth and dispersion were also restricted, which allowed a low allergen exposure and clinical, stabilized condition.

PB was collected into serum and lithium–heparin separating tubes.

IgE and eosinophils' determination

Serum total IgE was measured using a commercial kit (Coat-A-Count[®] Total-IgE IRMA, DPC[®], Los Angeles, CA, USA) based on an immune-radiometric assay of the solid phase and according to the manufacturer's guidelines. Concentration was determined by comparison with provided calibrators. Haemograms were performed in order to count blood eosinophils.

PBMC isolation and *in vitro* T cell stimulation

PBMCs were separated by gradient density using Biocoll (1.077 g/mL) (Biochrom AG, Berlin, Germany) from lithium–heparin tubes and 3×10^6 PBMCs per well (24-well plate) were stimulated *in vitro* (48 h, 37 °C and 5% CO₂), in an AIM-V serum-free medium (Gibco Brl, Invitrogen, Carlsbad, CA, USA) with 1 $\mu\text{g}/\text{mL}$ of LPS-free anti-CD3/anti-CD28 (granted from Swiss Institute of Allergy and Asthma Research, SIAF, Davos, Switzerland), also setting a control without antibodies (basal condition).

mRNA isolation and reverse transcription reaction

mRNA isolation was carried out according to the Qiagen-RNeasy protocol (Qiagen, Valencia, CA, USA) and stored at -80 °C. RT-PCR was performed with ~ 1 μg RNA transcribed into cDNA with random hexamers and moloney

murine leukaemia virus reverse transcriptase (all from Fermentas GmbH, St. Leon-Rot, Germany). The reactions were performed in a Thermocycler (Mycycler, BioRad, Hercules, CA, USA) using the following steps: 10 min at 25 °C, 60 min at 42 °C, 10 min at 70 °C and a final hold at 4 °C. cDNA were stored at –80 °C for no longer than 2 weeks.

Real-time PCR

cDNA was amplified using Sybergreen mix according to the manufacturer's recommendations, on a 96-well PCR plate, and run on an IQ5 Real-Time PCR Detection System (all reagents and systems from BioRad). The following sequence of primers was referenced by SIAF: T-bet fwd GATGCGCCA GGAAGTTTCAT, T-bet rev GCACAATCATCTGGGTCACATT; GATA-3 fwd GCGGGCTCTATCAGAAAATGA, GATA-3 rev GCTCTCCTGGCTGCAGACAGC; FoxP3 fwd GAAACAGCAC ATTCCAGAGTTC, FoxP3 rev ATGGCCAGCGGATGAG; elongation factor-1 (EF-1) fwd CTGAACCATCCAGGCCAAAT, EF-1 rev GCCGTGTGGCAATCCAAT.

Real-time PCR conditions were as follows: stage 1: 2 min at 50 °C; stage 2: 3 min at 95 °C; stage 3: 15 s at 95 °C and 45 s at 60 °C (40 repeats).

The amount of FoxP3, GATA-3 and T-bet mRNA expression was normalized with endogenous control EF-1 (ΔC_t values) and the relative quantification and calculation of range of confidence were performed using the comparative threshold cycle ($2^{-\Delta\Delta C_t}$) method (relative gene expression), as described by Livak and Schmittgen [18]. All amplifications were carried out at least in duplicate.

Statistical analysis

Statistical analysis was performed using the SPSS 12.0 software package. Data were analysed using either ANOVA (performing the Tukey HSD *post hoc* tests), Student's *t*-test (normality test passed with Kolmogorov–Smirnov) or the Mann–Whitney non-parametric test (normality test failed). Pearson correlation analysis was also performed between the variables studied. Significance was defined for P -value < 0.05.

Results

Twenty-two asthmatic patients presented positive SPTs to at least one of the common aeroallergens tested and were classified as allergic. Asthmatics presented normal mean percentual values of FEV₁ (90±23), and in five individuals, these values were below 60% in accordance with a diagnosis of severe asthma. Within the asthmatic group, six patients presented high FENO values.

Age, sex, lung function, serum total IgE, blood eosinophils and FENO from elderly asthmatic patients and healthy non-allergic non-asthmatic elderly volunteers are summarized in Table 1. Allergic patients presented higher serum IgE values and blood eosinophil counts and the differences observed between allergic and non-allergic, for serum total IgE, were statistically significant.

The FoxP3 and GATA-3 ΔC_t values under basal conditions were reduced in asthmatic patients when compared with the control group, while the T-bet ΔC_t values were similar in both the groups studied (Fig. 1). Comparing the studied groups for FoxP3, GATA-3 and T-bet relative gene expression after *in vitro* stimulation, higher values (fold increase average±standard deviation) were observed in controls (healthy individuals) than in asthmatic patients (10.2±6.8 vs. 4.8±3.8, 2.4±2.9 vs. 1.7±0.9 and 3.3±2.1 vs. 2.1±1.5, respectively). Asthmatics showed lower relative mRNA expression values of FoxP3 and T-bet. These differences among groups were statistically significant (P <0.05) (Figs 2a, c and d).

Subdividing the asthmatic group into non-allergic and allergic patients, the main statistically significant differences were once more for FoxP3, with lower and similar relative mRNA expression values in allergics (4.8±3.0) and non-allergics (4.8±4.3), contrasting to higher values in healthy individuals (10.2±6.8) (P <0.05) (Fig. 2b). However, the GATA-3 and T-bet values were similar without statistical significance when allergic and non-allergic patients were considered and compared with the control group (1.4±0.8 and 1.8±1.0 vs. 2.4±2.9, respectively, for GATA-3; 1.9±1.5 and 2.2±1.6 vs. 3.3±2.1, respectively, for T-bet).

Table 1. Population description

	Allergic asthmatics patients (n = 22)	Non-allergic asthmatics patients (n = 10)	Controls (n = 17)
Gender: female/male	15/7	6/4	10/7
Age (years; mean±SD)	72±5	71±4	78±7
Serum total IgE (kU/L; mean±SD)	313±284♦	116±101♦	66.7±38.1
Blood eosinophils (cells/μL; mean±SD)	237±223	168±116	154±88.9
Lung function FEV ₁ (L/min) (% mean predicted; mean±SD)	90±23.5	90±21.1	102.8±24.0
FeNO (p.p.b.; mean±SD)	25.7±22.2	29.2±28.3	20.1±14.3

Multiple comparisons were performed using ANOVA analysis and differences in P -values ≤ 0.05 were considered significant.

♦ P <0.05.

FENO, fractional concentration of exhaled nitric oxide; FEV₁, forced expiratory volume in the first second; p.p.b., parts per billion; SD, standard deviation.

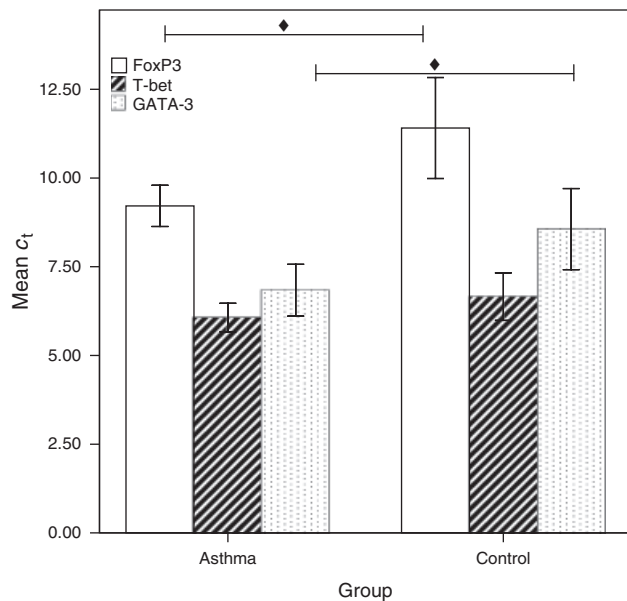


Fig. 1. FoxP3, T-bet and GATA-3 ΔC_t values for asthma patients ($n = 32$) and healthy control ($n = 17$) groups. The results are for ΔC_t under the basal condition after elongation factor-1 (EF-1) normalization and statistical significance is indicated by ♦ ($P < 0.05$) after performing *t*-test analysis. FoxP3, T-bet and GATA-3 mean values \pm standard deviation, control vs. asthma: 11.4 ± 2.7 vs. 9.2 ± 1.6 , 6.7 ± 1.2 vs. 6.1 ± 1.1 and 8.6 ± 2.1 vs. 6.8 ± 2.0 .

A positive correlation was found between total IgE and GATA-3 expression with statistical significance ($r = 0.382$, $P = 0.041$) in the asthmatic group (Fig. 3a). On the contrary, there was no correlation for the other transcription factors (FoxP3 and T-bet) with IgE values (Figs 3b and c) or other clinical features (data not shown).

Taking into account that increased levels of IgE production are associated with higher GATA-3 gene expression and lower T-bet gene expression, thus affecting the balance between Th2 and Th1 cells, the T-bet/GATA-3 ratio was also assessed using values after stimulation. When ratios between these investigated genes were analysed, statistical differences were observed between asthmatics and controls for the T-bet/GATA-3 ratio, with lower mean values in the patient group (1.5 ± 1.3) than in the controls (2.8 ± 3.3) ($P = 0.038$) (Fig. 4a). Considering that the balance between Treg/Th effector cells can also regulate the degree of inflammation in asthma, the Treg/Th2 ratio (FoxP3/GATA-3) was used additionally (Fig. 4b). Asthmatic patients presented a slightly reduced ratio (5.2 ± 6.6) when compared with healthy control subjects (8.8 ± 6.9) ($P > 0.05$) (Fig. 4b).

Discussion

This study was carried out to evaluate Th1-, Th2- and Treg-related lineage-specific gene expression, in long-lasting asthma. Asthmatics tend to demonstrate a decline in FEV1, which is related to the disease's progression, but can also be

affected by both genetic and environmental factors [2, 19]. Most of the patients had controlled asthma with lung function and FENO values within the normal range, despite the long disease duration [1]. It was also ensured that the healthy control subjects were free from atopy by performing SPTs despite the absence of symptoms.

Although IgE levels decrease with age, IgE-mediated allergy can affect 75% of elderly asthmatics, suggesting that the Th2 phenotype can still be dominant in this group. Th2 cells and GATA-3 play an important role in allergic inflammation and asthma, and induce IgE production [19]. The asthmatic patients presented high levels of total IgE (Table 1) when SPT was positive. On the contrary, the T-bet gene expression and Th1 pattern, along with the IFN- γ production, are usually associated with non-allergic asthmatics and healthy subjects. Although eosinophil infiltration is the main feature of both allergic and non-allergic asthma [7, 19], it is known that the overproduction of Th2-type cytokines can regulate antigen-induced eosinophil survival, leading to an increase of eosinophils in the allergic patients studied.

The values of GATA-3 gene expression of asthmatics were low under basal conditions and remained low after *in vitro* stimulation, in contrast to what was expected according to data for younger asthmatic groups who present an increase in GATA-3 expression [20]. The normal process of GATA-3 expression after *in vitro* stimulation is probably repressed in these patients, who seem to maintain the ability to produce high levels of IgE. The low grade chronic pro-inflammatory status in elderly (inflamm-ageing) probably plays a role in the relative bias towards Th2 immunity reported in senescence affecting both patients and healthy individuals [21] and reducing the differences in GATA-3 expression between the two groups analysed both in basal and in activated states. Nevertheless, a significant positive correlation was demonstrated between total IgE and GATA-3 expression ($r = 0.382$, $P = 0.041$) (Figs 1, 2c and 3a). This finding emphasizes the close relationship between IgE and GATA-3 expression and underlines the importance of this transcription factor as an inducer of IL4/IgE production even in elderly patients. A statistical difference between asthmatics and controls for the T-bet/GATA-3 ratio (Th1/Th2 ratio), with lower mean values in the patient group ($P = 0.038$), was also observed (Fig. 4a), which also emphasizes the Th2 role in asthma, where these cells are increased [19]. Therefore, the reduced ratio in Treg/Th2 cells observed can also contribute to the persistence of inflammation in long-lasting asthma (Fig. 4b).

The T-bet values, after *in vitro* stimulation, were significantly lower in asthmatics when compared with the control group (Fig. 2d). When considering both allergic and non-allergic groups, the GATA-3 and T-bet values were slightly lower, without statistical significance, when compared with the control groups. The atopic status does

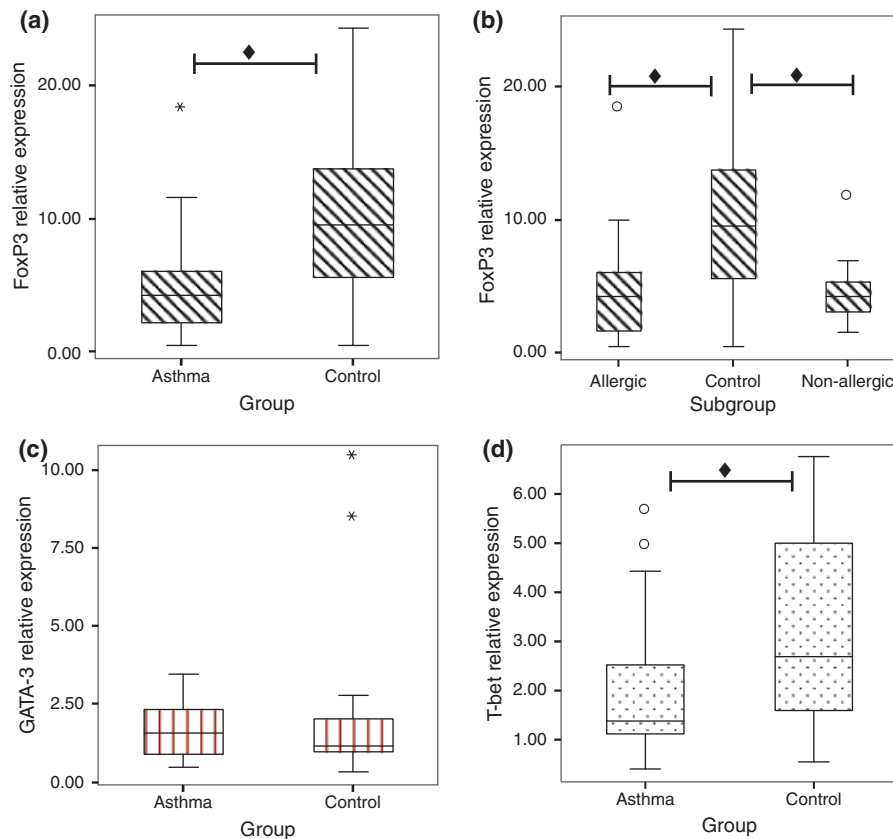


Fig. 2. FoxP3 (a and b), GATA-3 (c) and T-bet (d) relative gene expression levels for asthma patients ($n = 32$) [non-allergic asthma ($n = 10$) and allergic asthma ($n = 22$)] and healthy control ($n = 17$) groups. The results are expressed in fold increase ($2^{-\Delta\Delta C_t}$) comparing basal with activated conditions, and statistical significance is indicated by ♦ ($P < 0.05$) after performing a *t*-test and ANOVA analysis. Box plot graphs display the median (line within the box) and interquartile range (edge of the box). FoxP3, GATA-3 and T-bet fold increase mean values \pm standard deviation, control vs. asthma: 10.2 ± 6.8 vs. 4.8 ± 3.8 , 2.4 ± 2.9 vs. 1.7 ± 0.9 and 3.3 ± 2.1 vs. 2.1 ± 1.5 ; and non-allergic asthma vs. allergic asthma for FoxP3: 4.8 ± 4.3 vs. 4.8 ± 3.0 . *Extreme values; °outliers.

not introduce any adjustment in elderly patients. In fact, a decrease in the T-bet values has already been reported in severe asthma associated with airway remodelling affecting younger patients [19, 20]. In spite of this, we should emphasize again that this study was performed in elderly patients and the results should be interpreted considering the changes related to the immunosenescence process. Individuals aged 65 years or over present an age-associated shift towards the Th2 cytokine profile, affecting the typical atopic phenotype. Furthermore, in the elderly, there is a decline in the naïve strains while the sensitized cells become predominant [22].

T cell activation was induced with anti-CD3/anti-CD28, allowing naïve cells to become activated and to receive signals from other cells, which modulate the expression of T-bet, GATA-3 and FoxP3 genes. Cytokine environments induce a preferential expression of T-bet or GATA-3 and a Th1 or a Th2 dominant response with the release of their cytokines. The reduction of naïve cells in the elderly can explain the impaired development of these two differentiated phenotypes [23]. It should not be ignored that these results could be reinforced hypothetically with the mea-

surement of cytokine like IL-10, IL-4 or IL-13 in the culture supernatant. However, it is now recognized that IL-10 is produced by Th1, Th2, Th17 and Treg cells, while Th1 can produce IL-13 [24]. These recent findings argue for much more flexibility in cytokine production and support the controversial usefulness of their determination [24, 25]. The Foxp3 transcription factor, which can suppress the expression of T-bet and GATA-3 both with a reciprocal role, modulates the immune response when increased [14, 26]. Regulatory cells are supposed to reduce inflammatory responses in several diseases, including asthma. The Treg cell count is low in allergic patients and some therapeutic approaches such as specific immunotherapy can restore their normal values [20, 27]. The increased FoxP3 expression was relevant both in controls and in asthmatics upon activation, even when the two groups of patients were considered (Figs 2a and b), which might explain the low expression of GATA-3 and T-bet genes (Figs 2c and d). However, healthy individuals showed significantly higher FoxP3 expression values following activation when compared with asthmatics, as demonstrated previously by Provoost et al. [28] in

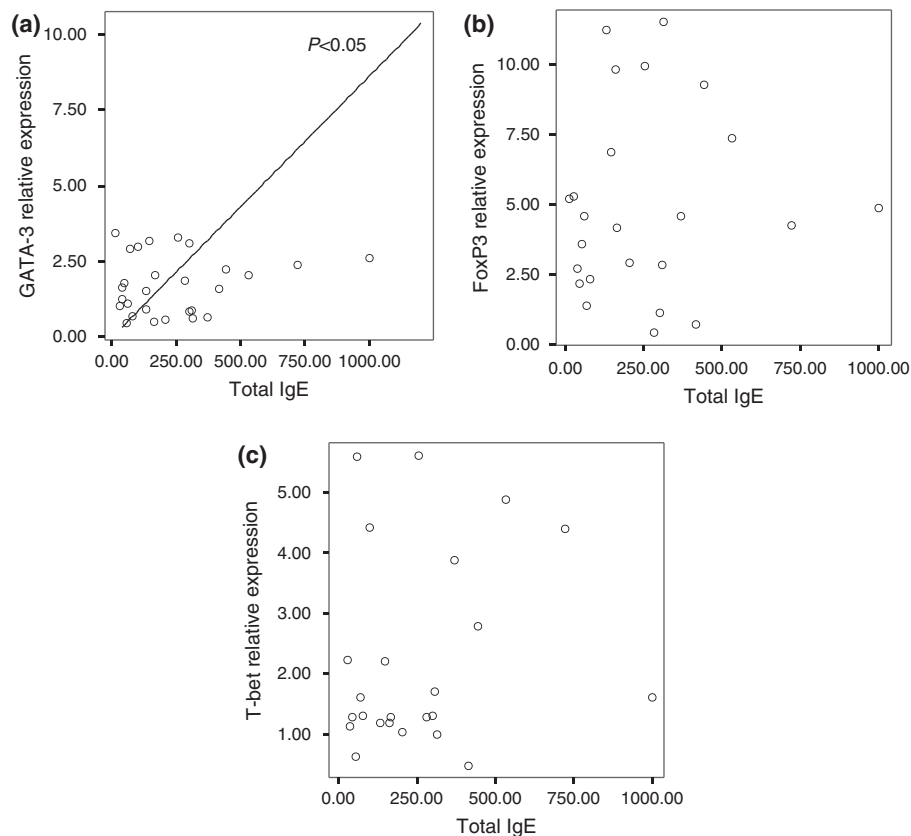


Fig. 3. Significant correlation between GATA-3 and total serum IgE levels (kU/L) in patients with asthma ($n = 28$) (a, Spearman correlation test, $r = 0.382$; $P = 0.041$). FoxP3 and T-bet relative gene expression values and total serum IgE levels (kU/L) in patients with asthma ($n = 28$) without a correlation (b, Spearman correlation test, $r = 0.032$; $P = 0.871$; c, Spearman correlation test, $r = 0.133$; $P = 0.499$). P -values were calculated after Pearson correlation analysis.

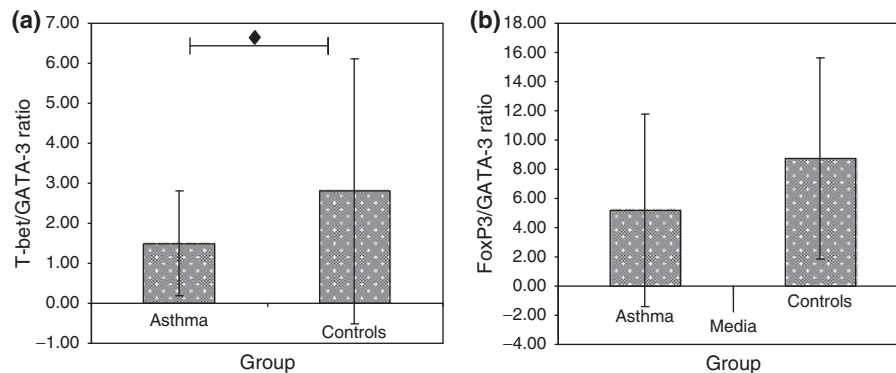


Fig. 4. Asthma ($n = 32$) and control ($n = 17$) group T-bet/GATA-3 ratio (a) and FoxP3/GATA-3 ratio (b). Bars show means [mean \pm standard deviation: (a) 1.5 ± 1.3 vs. 2.8 ± 3.3 , $P = 0.038$ and (b) 5.2 ± 6.6 vs. 8.8 ± 6.9 , $P > 0.05$]. P -values were calculated as a result of Student's t -test analysis.

younger groups. This lower value in asthmatics can provide an explanation for the sustained inflammatory process reported in long-lasting asthma.

The underlying chronic inflammatory state in the elderly, with high circulatory pro-inflammatory cytokines, is an additional stimulus for FoxP3 expression in this age group, narrowing the difference between patients and the healthy population.

Recent data demonstrate that the transcription factors for Th1, Th2 and Th17 cells, GATA-3, T-bet, ROR γ t and

FoxP3, respectively, can be co-expressed in some Tregs and exist *in vivo* [25]. Therefore, through this work, the hypothesis that these Treg cells may become effector cells and participate in normal immune response cannot be excluded. A better understanding of the reciprocal modulation of these three cell phenotypes that control the immunoinflammatory process is key in order to implement future therapeutic approaches.

In conclusion, efforts to improve asthma care and reduce the mortality have evolved from an understanding

of the inflammatory basis of asthma. Older people with asthma received low priority regarding research and interventions. Treatments that ameliorate asthma symptoms, including immunotherapy and steroids, which could restore Treg cell function, have deserved a better attention in the scientific community. This study demonstrates that elderly asthmatic patients also present a reduction in Treg cells. In addition, despite their low values of T-bet and GATA-3 expression, the ratio of expression of these two transcription factors shows a T-bet deficiency while GATA-3 expression increases in parallel to the IgE value. An improved understanding of the T cell network involved in long-lasting asthma affecting elderly allows considering new therapeutic approaches directed towards increasing FoxP3+Treg and decreasing GATA-3 cells also in elderly patients.

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